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Pollinator behavior and resource limitation maintain honest floral signaling

Knauer, Anina C ; Kokko, Hanna ; Schiestl, Florian P

Abstract: In many communication systems, signal receivers profit from honest signals that indicate the signaller's quality, whereas low-quality signallers should profit from cheating. Under such a conflict of interests between signallers and signal receivers, the maintenance of honest signals presents a puzzle. In theory, honesty can represent an evolutionarily stable strategy, but the actual mechanisms have been studied in few systems only. Here, we investigate honest signalling in a plant species, *Brassica rapa*, that advertises nectar volumes to pollinators by two honest floral signals; corolla size and the floral volatile phenylacetaldehyde. In a series of experiments we tested for physiological constraints and pollinator behaviours related to honest floral signals and nectar volume, incorporated the result into a mathematical model, and verified its predictions experimentally. While honest floral signals attracted pollinators, the bees' flower visitation time depended on nectar volume and was associated with the number of seeds that flowers developed. Furthermore, honest floral signals and seed set without pollen limitation both increased after soil fertilisation, indicating nutrient limitation in these traits. The mathematical model which incorporates these findings showed that honest signalling in *B. rapa* can be maintained by a combination of pollinator behaviour and resource limitation causing differential benefits of nectar production. The study demonstrates how honest floral signalling can evolve as a stable strategy in a plant species.

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Functional Ecology

Pollinator behaviour and resource limitation maintain honest floral signalling

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ABSTRACT

1. In many communication systems, signal-receivers profit from honest signals that indicate the signaller's quality, whereas low quality signallers should profit from cheating. Under such a conflict of interests between signallers and signal-receivers, the maintenance of honest signals presents a puzzle. In theory, honesty can represent an evolutionarily stable strategy, but the actual mechanisms have been studied in few systems only.
2. Here, we investigate honest signalling in a plant species, *Brassica rapa*, that advertises nectar volumes to pollinators by two honest floral signals; corolla size and the floral volatile phenylacetaldehyde. In a series of experiments we tested

for physiological constraints and pollinator behaviours related to honest floral signals and nectar volume, incorporated the result into a mathematical model, and verified its predictions experimentally.

3. While honest floral signals attracted pollinators, the bees' flower visitation time depended on nectar volume and was associated with the number of seeds that flowers developed. Further, honest floral signals and seed set without pollen limitation both increased after soil fertilization, indicating nutrient limitation in these traits.
4. The mathematical model which incorporates these findings showed that honest signalling in *B. rapa* can be maintained by a combination of pollinator behaviour and resource limitation causing differential benefits of nectar production. The study demonstrates how honest floral signalling can evolve as a stable strategy in a plant species.

KEYWORDS: *Bombus terrestris*, *Brassica rapa*, cheating, floral scent, flower size, pollination

INTRODUCTION

Plants signal to their animal pollinators to advertise rewards and in return receive directed pollen transfer between individuals (Schiestl & Johnson, 2013). Because rewards are normally concealed within flowers, pollinators rely on floral signals, like colour, size and scent, when choosing plants for visitation. Floral signals that are correlated with floral rewards and therefore considered to be honest can benefit pollinators by directing them to rewarding flowers (Howell & Alarcon, 2007). While honest floral signals among the flowers of one plant individual (e.g. change of signals in pollinated flowers with lower reward) can also benefit the plant by directing pollinators to receptive flowers while enhancing overall pollinator attraction (Benitez-Vieyra et al., 2006; Brito et al., 2015; Takashi T Makino & Ohashi, 2017), honest signalling among plant individuals within a population is more difficult to explain (Schaefer et al., 2004). Low-quality individuals, who offer little reward to pollinators, can cheat by producing strong signals to achieve high visitation rates by pollinators. Especially if nectar production is costly, cheating could be advantageous for plants, provoking a conflict between plants and pollinators.

Despite the apparent temptation to cheat, various plant species display honest floral signals that positively correlate with nectar or pollen amounts (Armbruster et al., 2005; Jose M. Gómez et al., 2008; M. Stanton & Young, 1994). Honesty could be maintained by several different mechanisms. First, signals can be so-called indices, in which case they are intrinsically linked to the signaller's quality constraining the possibility for cheating (flowers without rewards) and dishonesty (signals not correlated to rewards). Second, honest signalling can be stable when individuals do not profit from deviating from honesty (Belsare et al., 2009; Cohen & Shmida, 1993; Sun et al., 2018). This is expected to evolve when low-quality individuals experience higher costs and/or lower benefits from a high signal than do high-quality individuals, which prevents them from cheating (Grose, 2011; Higham, 2014) (Figure 1).

Honest floral signalling can be stable, as demonstrated by Cohen and Shmida's mathematical model (1993), if the following four conditions are met: 1. variable resource availability in flowers caused by variable total resource allocation to inflorescences between individuals of the same plant population, 2. resource limitation of the signal and the reward causing a trade-off between these traits (adjustment of these traits cannot be optimized simultaneously as an increase in one causes a decrease in the other), 3. pollinator-mediated selection on floral signals,

and 4. pollinator-mediated selection on floral reward (Figure 2A). Under these conditions, despite the trade-off between signal and reward, these traits are positively correlated as individuals optimize their fitness by allocating a specific proportion of the resource (p) to the signal and the remaining part ($1-p$) to the reward. In combination with variable resource availability in flowers among plant individuals, this causes an overall positive correlation in the population.

Signals and nectar can cause physiological (Ordano & Ornelas, 2005; Pyke, 1991; Vasconcelos & Proença, 2015) or ecological costs to plants (e.g. increase the conspicuousness and attractiveness to herbivores) (Kessler et al., 2015; Knauer & Schiestl, 2017; Ramos Castro & Schiestl, 2020), but they can also be under pollinator-mediated selection (Kulbaba & Worley, 2012; Sandring & Agren, 2009; Sletvold et al., 2010). Once a pollinator has landed on an inflorescence, it can consume and assess floral rewards and adjust its behaviour accordingly. Revisitation of a plant, the number of flowers that pollinators visit and probing time per flower can all be adjusted, benefitting plants with large nectar volumes (A. Brandenburg et al., 2012; Cresswell, 1999; Galen & Plowright, 1985; Kadmon & Shmida, 1992; T. T. Makino & Sakai, 2007; Mitchell, 1993; Real & Rathcke, 1991). Large floral display, in contrast, can benefit plants through increased pollinator attraction (Armbruster et al., 2005; Byers et al., 2014; Gervasi & Schiestl, 2017). The pollinator's preference for certain signals can be either innate or learned, where learning is especially relevant for generalist pollinators that forage on various plant species with different signals. Laboratory learning assays showed that bumble bees can learn to distinguish between honest and dishonest floral signals and develop preferences for honest floral signals over a rather short time (Burdon et al., 2020; Knauer & Schiestl, 2015), but it is still unknown if similar learning capacities exist in natural environments with many different plant species being present (Leonard & Francis, 2017).

To test the model presented by Cohen and Shmida (1993), we used the field mustard, *Brassica rapa*, which emits two floral signals that honestly indicate differences in average nectar amount between individuals: corolla size and the emitted amount of the floral volatile phenylacetaldehyde (Knauer & Schiestl, 2015). In some cases, honesty is guaranteed if the signals are directly emitted by the rewards themselves (physiological constraints) (Dobson et al., 1999; Raguso, 2004), but as nectar does not emit any scent in *B. rapa*, an unmodifiable relationship between nectar volume and scent can be excluded as the cause of honesty in this

species (Knauer & Schiestl, 2015). Also, pleiotropy (genetic constraint) is an improbable source of signal-nectar correlation in *B. rapa* as nectar production and honest floral signals involve supposedly different metabolic pathways (Borghi et al., 2017). The mechanism maintaining signal-reward correlation in *B. rapa* is therefore still unknown. Here we ask if in a natural environment the bees' preference for a floral signal depends on its honesty in the plant population, and if in *B. rapa* honest signalling is an evolutionarily stable strategy maintained through differential costs or benefits of signal and/or nectar production. We test for the predictions 2 to 4 made by Cohen and Shmida (1993) by addressing the following questions: 1) Is there pollinator-mediated selection for larger honest signals and nectar volumes? 2) Are there shared resource limitations (physiological costs) in nectar and signal production? Based on the experimental results, we then present an extended mathematical model that shows how honest signalling can be maintained. The components and predictions of this model are then tested in another series of experiments.

MATERIALS AND METHODS

Study system

Field mustard, *B. rapa*, is a self-incompatible, annual or biennial herb (Watanabe et al., 2000) with a generalised pollination system. Especially different bee species are important pollinators in terms of visitation rate and pollination efficiency (Rader et al., 2009; Sahli & Conner, 2007). *B. rapa* seeds were collected in a natural population from about 100 individuals (population size over 1000 individuals, Maarssen, the Netherlands) and grown under standardised light, temperature, soil and watering conditions in a greenhouse (light period: 16 h, temperature: 22°C, 400 mL pots with standardized soil, watering by flooding cultivation tables twice a day for 30 min each). All plants were treated every second week with the pesticides Kendo and Thiovit until start of flowering. The bumble bee *Bombus terrestris* colonies used in this study were purchased from Andermatt Biocontrol (Andermatt, Switzerland) and the hives were kept in a flight cage (3 x 1 x 1 m). For each experiment one hive was used. Bumble bees were fed on pollen (purchased directly from beekeepers) and sugar solution (Apiinvert, Südzucker AG, Ochsenfurt). Additionally, we exposed the bees to 20 to 30 flowering *B. rapa* plants for at least 3 h before experimental use.

Use of honest signals by bees in a natural environment

To test the prediction that bees prefer honest floral signals over dishonest ones in a natural foraging environment, we set-up a field experiment which was composed of a training and a testing phase. For the training phase we placed 28 *B. rapa* plants on a meadow in the botanical garden of Zürich for 3.5 h during six days. Every day a different set of plants were used (168 plant in total), which were placed with a distance of 75 cm. During this training phase wild pollinators were allowed to visit flowers and learn to associate signals with rewards. Each day plants were assigned to one of the following treatments: a) natural scent emission; plants emitting phenylacetaldehyde as an honest signal; b) scent augmentation with various different phenylacetaldehyde amounts in part of the plants; plants emitting phenylacetaldehyde as a dishonest signal as a consequence (see below for description of scent manipulation). These two treatments were alternated between days, three times each, during sunny and warm days.

After the 3.5 h training phase, all plants were removed and immediately replaced with five test-plots of four plants each. During the testing phase, these plants were used to test the bees' preference for phenylacetaldehyde after the different training treatments. We augmented phenylacetaldehyde emission in two plants per plot whereas the other two plants emitted natural amounts of the compound (no manipulation). Bee visits to plants with high and low phenylacetaldehyde emission were recorded throughout the 30 min that plots were maintained. Although *B. rapa* plants were also visited by syrphid flies, we focused on bees as they represent the main and most efficient pollinators. Within each plot, plants with the same scent treatment were placed side by side with a distance of 0.5 m; the distance between plots was 2 m. Further, to control for genetic background, we used representatives of the same full sib families at the same positions per plot on subsequent experimental days (after different training treatments). All plants used during this experiment were in full flower (mean \pm s.e. flower number: 36.7 ± 1.2) and flower number did not differ between the two scent treatments in the testing phase for any experimental day (data not shown).

We augmented the emission rate of phenylacetaldehyde using scent application by grey rubber septa (Supleco, Bellefonte, PA, USA) (Huber et al., 2005) which were fixed on the main inflorescence branch with thread. Rubber septa were soaked in solutions of phenylacetaldehyde in dichloromethane for 1 h and afterwards

dried for 5 h before experimental use to obtain stable emission rates. For the training treatment b) we used four different solutions (0, 1, 2 and 3 $\mu\text{l mL}^{-1}$ phenylacetaldehyde in dichloromethane). Septa soaked in these solutions (7 septa per concentration) were assigned randomly to plants to disrupt honesty. For the scent augmentation in plots we used a solution of 3 $\mu\text{l mL}^{-1}$ phenylacetaldehyde in dichloromethane. Septa prepared in this way emitted phenylacetaldehyde in a concentration realistic for *B. rapa* inflorescences (Knauer & Schiestl, 2015). Control septa which were used for all plants with natural scent emission were soaked in pure dichloromethane.

To test for differences in the effect of scent augmentation after different training treatments, we fitted a generalized mixed model with a Poisson distribution. The number of visits was included as the response and scent manipulation treatment in the testing phase (augmented or not), the training treatment (honest or dishonest) and the flower number as explanatory variables. The interaction term training treatment x testing treatment was included to test for different pollinator preferences for phenylacetaldehyde after different training treatments. Day and plot were included as random effects in the model (plot nested in day).

As the interaction between training and testing treatment was significant, we fitted a separate model for each learning treatment to obtain estimates for the effect of the phenylacetaldehyde augmentation on bee attraction after different training treatments (identical model as described above but excluding training treatment as explanatory variable). All statistics were done with R 4.0 (R Core Team, 2020).

Honest signalling and selection on honest signals and nectar volume

To measure honest signalling and selection on honest signals and nectar volume as predicted by Cohen and Shmida's model (1993), we conducted a plot experiment exposing 36 *B. rapa* plants (6 x 6, 40 cm distance between plants) to 12 bumble bees in an outdoor cage (3 x 3 x 2 m). We measured corolla size, floral scent and nectar volume of all plants one day before pollinator exposure. Additionally, total number of flowers that opened during pollinator exposure were counted. Each plot was kept in the cage for two subsequent sunny days, and each day bumble bees were released to visit plants twice (about 15 min, three bees at the time). All bees were marked with a dot to avoid multiple usage of individuals during different exposure phases. In total we conducted three replicates leading to a total sample

size of 108 plants and 36 bees. After fruit development was completed, the number of seeds per individual was counted as a proxy for female fitness.

Nectar volume was measured in three flowers per individual with 5 μ L micropipettes (Blaubrand, Wertheim, Germany) to calculate mean nectar volume per flower. In *B. rapa* nectar volume is highly correlated with nectar sugar amounts ($R = 0.75$, $P < 0.001$; Knauer & Schiestl, 2015) and therefore a good estimate of reward amount.

Petal length and width were measured in three fully opened flowers per individual. Means of petal length and width were used to estimate the corolla size per flower as $\pi \times length \times width$.

For scent collection from inflorescences we used the push-pull headspace collection method (Schiestl et al., 2014). Inflorescences were enclosed in glass cylinders (dimensions: 5 cm diameter, 25 cm height; all glass cylinders were treated previously with sigmacoate. The bottom of the cylinder was closed with a teflon plate with a central hole allowing for the insertion of the peduncle without injuring it. For volatile collection glass tubes filled with ca. 20 mg of Tenax TA (Tenax TA 60/80, Supelco, Bellefonte, PA, USA) were inserted into a small opening in the cylinder and attached to a vacuum pump (DC06/04/20F, Fürgut GmbH, D-88459 Tannheim) with a silicon tube. Air was pulled through the Tenax tubes for 1 h at a flow rate of 150 mL/min⁻¹. After passing the tube, the air was circulated back (with the same flow rate) to the glass cylinder through another Tenax tube (Tenax GR 60/80, Scientific Instrument Services, Old York, NJ, USA), which was inserted through a second opening, to clean the incoming air. The number of flowers inside the cylinder was counted to calculate volatile amounts per flower. All collections took place between 11⁰⁰ and 15⁰⁰ hrs in the greenhouse. After scent collection the Tenax tubes were stored at -30°C until chemical analysis. For the analysis of floral volatiles, gas chromatography with mass selective detection (GC-MSD) was used as described in Knauer & Schiestl (2015). Phenylacetaldehyde was BoxCox-transformed (with $\lambda = 0.2$) after quantification.

To test for signal honesty, we calculated Pearson's correlation between nectar volume and floral signals (for corolla size and phenylacetaldehyde separately). To test for selection on floral signals and nectar volume we fitted a model with relative female fitness as response and the nectar amount, corolla size and phenylacetaldehyde emission as explanatory variables. Within replicates, we

standardized floral traits by z-transformation. The number of flowers was included into the model to control for display size. Before statistical analysis, relative female fitness was estimated by dividing the seed set of each individual by the replicate mean. For the calculation of P values, this estimate of relative fitness was $\ln(1+x)$ transformed to obtain homogeneity of variance and approach normal distributions of residuals (Lande & Arnold, 1983; Mitchell-Olds & Shaw, 1987).

Resource limitation

The mechanism suggested by Cohen and Shmida (1993) includes resource limitation in reward and honest signal by the same resource causing a trade-off between the two traits. To test for resource limitation by soil nutrients in honest floral signals and nectar volume we measured the response of these traits to the application of fertilizer. 36 *B. rapa* plants were grown in 400 mL of standardized soil in the greenhouse and assigned to the following two treatments to the plants in a row alternately: 1) application of 0.5 g fertilizer; 2) no fertilization. We used long term fertilizer (Osmocote Exact Standard 3-4 months, 16% nitrogen, 9% phosphate, 12% potash, 1.2% magnesium), which is solid and releases nutrients for six months at constant rate. Therefore, application of fertilizer was done only once when inflorescences started developing (six weeks after sowing). When plants were in full flower, we measured corolla size, phenylacetaldehyde emission and nectar volume (measurement of floral traits described above). Further, we hand-pollinated 9 flowers per individual with pollen from three donor plants (three flowers per pollen donor) to calculate mean number of seeds per fruit after fruit ripening. Incompatibility reactions were identified as specific crossings not producing fruits (one out of three pollen donors did not produce seeds in a specific recipient plant) and excluded from the analysis.

An equivalent treatment was used to test for water limitation. 36 plants were assigned to the following two treatments: 1) high water availability by watering twice a day; b) low water availability by watering once a day. Watering was done by flooding greenhouse tables for 30 min. The treatments were commenced when inflorescences started developing. When plants were in full flower, we measured floral traits and seed development after hand-pollination as described above.

Linear models were used to test for differences in floral traits between fertilization or water treatments. A separate model was fitted for each trait per experiment.

Model of signalling nectar rewards

As predicted by Cohen and Shmida (1993) we found positive pollinator-mediated selection on nectar volume and honest floral signals. In contrast, reward and signals were not limited by the same resources. Instead, we found nutrient limitation in honest signals and the number of seeds that flowers could develop maximally after hand-pollination. To test if honesty could nevertheless be stable, we measured how honest floral signals and nectar volume shape pollinator behaviour and how this affects female plant fitness (see below). We subsequently included these findings in a mathematical model to calculate net benefits of plants following different strategies regarding the resource allocation to the different components in the model.

Equivalent to Cohen and Shmida's model, our model assumes that plant individuals vary in their resource availability in flowers, A . As our data suggests a trade-off between seed development and honest signals (because of resource limitation in honest floral signals and seed development), we assume that one part of a plant's strategy is the proportion p allocated to honest floral signals S , which become $S = pA$. The maximum number of developing seeds (per fruit) is then $D_{\max} = (1-p)A$, but the maximum will only be reached if pollinator visitation time is sufficiently long.

Because, in *B. rapa*, honest floral signals attracted bees to visit flowers, we model the number of visits per day V as $V = V_{\max}(1 - e^{-k_1 S})$. Here V_{\max} represents the maximal possible number of visits to inflorescences per day and hence reflects the total number of pollinators in the area of the plant population (the assumption of a limited number of pollinators calls for an asymptotic relationship), while k_1 is a constant defining the speed at which the asymptote is approached.

Each visiting pollinator spends a certain amount of time on the inflorescence. As the pollinator now has information on actual nectar volume, we assume this information to override the effect of the signal as a determinant of visitation time T . We assume constant consumption rates and that pollinators consume the whole nectar volume available; thus the relationship between flower visitation time and nectar volume is a linear function, $T = T_{\min} + k_2 N$. Here T_{\min} represents the minimal

flower visitation time that pollinators need to land and orientate themselves on flowers, and N is nectar volume.

The plant has to ‘decide’ its allocation to signalling, p , but also the amount of nectar to produce, N . We base fitness on the female function (see Discussion for reflections on the male part), with three components: a documented positive effect of flower visitation time (T) on the numbers of developing seeds, D ; a documented positive effect of the visitation rate (number of visits V) on the number of fruits that develop per day, F ; and a linear cost of nectar production that impacts plant fitness as a whole, W . These combine in the following way:

$$D = D_{\max}(1 - e^{-k_3 T}), F = F_{\max}(1 - e^{-k_4 V})$$

$$W = DF - C(N) = DF - k_5 N$$

where $C(N)$ is the (linear) cost of nectar production.

The calculation of W leads to a single best (fitness-maximizing) combination of values of N and p for each value of A (resource availability for flower production as a whole), which was assumed to vary by a factor of 10 (from 5 to 50). The parameters $k_1 \dots k_5$, V_{\max} , T_{\min} and F_{\max} were not fitted in a statistical sense to our data, but were chosen to be plausible, such that the observable set to values appears realistic for *B. rapa* (as indicated in Table S1 in the Supporting Information).

While the example shown can be used to examine the logic of honest floral signalling, we can also use the equations to see which features, if absent, will ‘break the model’ in the sense of removing honest signalling (the positive relationship that allows a pollinator to predict N based on S).

Measuring the different components in the model

Pollinator behaviour in response to nectar volume

This experiment aimed to assess the behavioural response of bumble bees to nectar volume. We placed 36 *B. rapa* plants (6 × 6, 40 cm distance between plants) in a cage (3 × 3 × 2 m) and released five bumble bees for 15 minutes. One day before the experiment we measured floral nectar volume, corolla size and phenylacetaldehyde emission (measurement as described above) for all plants. During pollinator exposure we recorded the number of flowers that bumble bees visited and the time they spent on the inflorescence for each visited plant. The mean time spent per flower was calculated as *(total time on inflorescence)/(number of visited flowers)*.

Data was analysed with multiple regressions with the nectar volume, corolla size and phenylacetaldehyde as explanatory variables. The visitation time per flower and the percentage of visited flowers were fitted as responses in two separate models.

Pollinator behaviour in response to honest signals

To test for pollinator attraction by honest floral signals, plant visitations in the selection experiment was analysed. We fitted a generalized linear model with a Poisson distribution, with the number of visits to individual inflorescences by bumble bees as response and corolla size and phenylacetaldehyde emission as explanatory variables. The number of flowers was included into the model to control for display size.

Effect of flower visitation time on female fitness

Because we found a positive association between floral nectar volume and visitation time by bumble bees, we tested for a positive effect of visitation time on female plant fitness. For that purpose, we allowed 18 *B. rapa* plants to be visited by one bumble bee each (18 bumble bees in total) in a netting (3 × 1 × 1 m). The entire visit was recorded by a camera (Sony handycam HDR-CX220E) and the visitation time for each visited flower was measured. After fruit ripening, we counted the number of seeds that visited flowers developed. Identification of flowers in videos was guaranteed by coloured markings on inflorescences. Before bumble bees were released to visit the experimental plants, we let them forage on two *B. rapa* plants to ensure substantial pollen load for pollination.

To test for an effect of flower visitation time on seed number, we used a generalized mixed effect model with a Poisson distribution. The response variable was the number of seeds and the visitation time was included as the explanatory variable; the plant individual was included as a random slope.

Effects of the number of visits to inflorescences on female fitness

As corolla size and phenylacetaldehyde emission positively affected bumble bee attraction in our plants, we measured the effect of the number of visits on female plant fitness. Plant individuals were exposed to one, two or three bumble bees successively in a netting (3 × 1 × 1 m). We measured 9 plants per treatment (27

plants in total) and each bumble bee was only used once in the experiment (54 bumble bees). We let each bumble bee first visit a pollen donor plant (a different plant for each bumble bee) before it was allowed to visit the target plant. The visit was considered as completed when bumble bees moved on to a third plant in the cage. For each experimental plant we counted the number of open flowers and, after fruit development, the total number of fruits and seeds.

To test for the effect of visitation rates on female fitness, we fitted a linear regression with the fruit set (number of fruits/number of flowers) as response and the number of visits as explanatory variables. Additionally, we tested for an effect on seed development by fitting a regression with the mean number of seeds per fruit as response.

Verification of the mathematical model

Because of the asymptotic relationship between nectar volume and female plant fitness, our model predicts a stronger effect on plant fitness after nectar removal than after nectar addition (see also Figure 5). To verify this feature of the model, we set up an experiment manipulating nectar volumes in 20 *B. rapa* plants. In each of these plants we removed nectar from all flowers of one spike and added this nectar to the flowers of another spike on a flower-to-flower basis (i.e. the nectar of one flower was transferred to another flower by a pipette). In a third spike nectar volumes were not manipulated. The flower number in the three spikes were equalized by cutting off surplus flowers, and the position of treatments within inflorescences were alternated between experimental plants. Also, all flowers were emasculated to preclude pollen collecting behaviour in bees. Before the experimental testing we set up a bumble bee colony and a population of about 30 *B. rapa* plants in a netting (3 × 1 × 1 m) to allow for associative learning in bumble bees and ensure sufficient pollen load for pollination. Subsequently one bee at the time was allowed to visit one spike of an experimental plant, while the other two were covered by nets. We measured the number of visited flowers and the mean visitation time per flower in each of these visits. Additionally, we counted the total number of seeds after fruit ripening in each spike.

The data was analysed by mixed effect models with the visitation time per flower, the number of non-visited flowers, or the mean number of seeds per flower as response and the nectar treatment (removed, doubled and non-manipulated nectar)

as explanatory variable. For the number of non-visited flowers we used a generalized model with an observation level random effect (OLRE) to cope with overdispersion (Harrison, 2014). The plant individual was included as random term in all models.

RESULTS

Use of honest signals by bees in a natural environment

The bees' preference for phenylacetaldehyde depended on the honesty of the signal. In total we observed 235 visits by bees: 220 by *Apis mellifera* and 15 by *Bombus spp.* Bee attraction by scent augmentation depended on the honesty of the signal during the training phase (significant learning treatment \times scent augmentation interaction: $\chi^2 = 8.28$, $P = 0.004$) (Figure 3). After bees had visited unmanipulated plants emitting phenylacetaldehyde as an honest signal, they showed a preference for augmented phenylacetaldehyde emission ($\chi^2 = 6.54$, $P = 0.01$). Plants with augmented phenylacetaldehyde emission received in total 1.7 times more visits by bees than unmanipulated plants (Figure 3). In contrast, after bees had visited manipulated plants emitting phenylacetaldehyde as a dishonest signal, bees did not show any preference for phenylacetaldehyde ($P = 0.14$) (Figure 3).

Honest signalling and selection on honest signals and nectar volume

Both corolla size and phenylacetaldehyde were correlated with nectar volume (corolla size: Pearson's $r = 0.38$, $P < 0.001$; phenylacetaldehyde: Pearson's $r = 0.55$, $P < 0.001$) (Figure 4). Corolla size, phenylacetaldehyde and nectar volume as well as flower number were all under positive directional selection (Table 1).

Resource limitation

The mechanism suggested by Cohen and Shmida (1993) includes a trade-off between honest signals and reward. Under their scenario, the two traits are nevertheless positively correlated because individuals vary in resource availability in flowers among plant individuals and always allocate a certain proportion p of the resources to the signal and the remaining part $(1-p)$ to the reward (see Fig 1 and Van Noordwijk & de Jong, 1986). If this mechanism applies, honest floral signals and reward should be limited by the same resource and increase when resource availability raises (but the positive correlation between reward and signals should not

be affected). Fertilization of *B. rapa* plants did not affect the amount of nectar per flower ($P = 0.83$), but it increased the emission of phenylacetaldehyde by 88% ($t = 2.11$, $P = 0.044$) and corolla size by 17% ($t = 2.13$, $P = 0.042$). Also, fertilized plants produced a 175% higher number of seeds per fruit after removing pollen limitation by hand-pollination ($t = 3.66$, $P = 0.001$).

Water availability, in contrast, increased nectar volume by 79% ($t = 4.41$, $P < 0.001$). Additionally, the emission of phenylacetaldehyde was increased by 16% ($t = 3.05$, $P = 0.005$), while corolla size was not affected ($P = 0.75$). Plant with high water availability further produced 59% more seeds per fruit after hand pollination ($t = 2.39$, $P = 0.024$).

Model of signalling nectar rewards

Given a trade-off between floral signals and seed development, plant maximize their female fitness when allocating part of the available floral resources to seed development and the remaining part to floral signals, with the optimal p declining mildly with A (Figure 5A). This decline suggests a *relative* reduction in signalling when A increases, but the response is so mild, that the absolute signalling effort, pA , increases with A (Figure 5B). Simultaneously, it is optimal for high- A individuals to produce more nectar (Figure 5B), leading to a positive covariation between signal and nectar, i.e. honesty (Figure 5C). Honesty clearly results from differential benefits: individuals with low A can never reach as high seed numbers D as those with high A , and assuming equal costs of nectar production, they profit less from increased nectar volumes and the resulting longer pollinator visits than individuals with high resource availability (Figure 5C and 5D).

The model needs all its components to produce honest signalling. Changing any of the functions to flat lines destroys honesty, though in different ways. If $V = V_{\max}$ regardless of the signal, nectar will be produced (and high- A individuals will produce more than low- A individuals), but there are no floral signals (p evolves to zero). If, on the other hand, we assume that $T = T_{\min}$ regardless of N , p will still follow a mild negative relationship to A , but since a higher nectar volume now will not entice a pollinator to stay for longer, nectar production will evolve to zero, and a positive signal S will no longer be associated with any positive reward for the pollinator. Over evolutionary time, we would then expect the relationship between S and V to disappear. Setting $D = D_{\max}$ regardless of T has the same qualitative effect as setting

T identical across values of *N*: there is again no incentive for nectar production for the plant, destroying honesty. Finally, if we assume $F = F_{\max}$ regardless of *V*, nectar production continues such that high-*A* individuals produce more, as this scenario assumes that the per-fruit productivity depends on the duration of pollinator visits; however, honesty is not maintained, as there is no incentive to produce any signals. Finally, if the trade-off between floral signals and seed development is uncoupled by simulating resource limitation by two different resources, nectar and signal production will both be maintained but the association between them is lost, resulting in dishonesty.

Measuring the different components in the model

Corolla size as well as the amount of phenylacetaldehyde per flower were positively associated with the number of visits to inflorescences by bumble bees (corolla size: $\beta = 0.006 \pm 0.002$, $z = 2.774$, $P = 0.006$; phenylacetaldehyde: $\beta = 0.03 \pm 0.01$, $z = 2.409$, $P = 0.016$). Additionally, we found a positive association between the number of flowers and pollinator attraction ($\beta = 0.02 \pm 0.003$, $z = 5.360$, $P < 0.001$).

Bumble bees spent more time on flowers with larger nectar volumes ($\beta = 10.2 \pm 2.7$, $t = 3.773$, $P = 0.003$) (Figure 6). In contrast, corolla size did not affect floral visitation time ($P = 0.27$) and phenylacetaldehyde even decreased the time ($\beta = -0.22 \pm 0.06$, $t = -3.552$, $P = 0.005$). Neither nectar volume nor honest floral signals had an effect on the percentage of flowers that bumble bees visited in an inflorescence (nectar volume: $P = 0.54$; corolla size: $P = 0.83$; phenylacetaldehyde: $P = 0.07$).

Both the number of visits and visitation time positively affected fitness. Each additional visit by a bumble bee increased the fruit set by $10.5 \pm 4.5\%$ ($t = 2.285$, $P = 0.031$). Interestingly, the number of seeds per fruit was not affected by the number of visits ($P = 0.19$), but increased with the visitation time per flower ($\beta = 0.15 \pm 0.03$, $\chi^2 = 25.8$, $P < 0.001$).

Verification of the mathematical model

To verify the mathematical model, we tested for the effect of nectar manipulation on bee behaviour and female plant fitness. Because of the asymptotic relationship between nectar volume and fitness, the above described model predicts differential

benefits of nectar volume and a stronger effect on plant fitness after nectar removal than after nectar addition (see also Figure 5). The removal of nectar increased the number of unvisited flowers ($\beta = 1.0 \pm 0.35$, $z = 2.860$, $P = 0.004$) and reduced bee visitation time per flower ($t = -2.695$, $P = 0.01$) and the number of developed seeds ($t = -2.841$, $P = 0.007$) (Figure 7). In contrast, the doubling of nectar volumes caused a significant increase in bee visitation time ($t = 2.829$, $P = 0.008$), but did not affect the number of unvisited flowers ($P = 0.23$) or seed number ($P = 0.67$) (Figure 7) (see also Appendix S1 and Figure S1 in the Supporting Information for further verification of the model).

DISCUSSION

Although honest signalling in plant-pollinator communication has been documented for various plant species (Armbruster et al., 2005; Jose M. Gómez et al., 2008; Knauer & Schiestl, 2015; M. Stanton & Young, 1994), it still remains a puzzle why low-rewarding individuals do not cheat, destroying signal honesty. Here we show that in honestly signalling plant populations, individuals benefit from high signal values by increased pollinator visitation rates irrespective of their reward value. Although this should at first sight lead to the breakup of honesty over time, honest signalling seems to be a stable strategy in *B. rapa*. We resolve this apparent contradiction by showing that honest signalling is maintained by a combination of pollinator behaviour and resource limitation. Individuals with low resource availability in flowers improve their fitness by producing small signals, given a trade-off between signals and seed development. Simultaneously, because of resource limitation in seed production, such individuals profit less from large nectar volumes and resulting long visitation times by bees compared to individuals with high resource availability. Thus, our study provides evidence that differential benefits maintain signal honesty in *B. rapa*.

In contrast to the prediction made by Cohen and Shmida's mathematical model (1993) our data does not support resource limitation in honest floral signals and rewards by the same resource. Although both nectar volume and the honest signal phenylacetaldehyde were reduced by water limitation, the effect of nutrient limitation on phenylacetaldehyde was much stronger. Consistent with these findings, we have modified the Cohen-Shmida model to one where honesty can be maintained despite the absence of a direct trade-off between signals and reward. In

line with our model, nectar removal from flowers caused shorter visitation times by bumble bees and a reduction in female plant fitness, while nectar addition increased visitation times but not fitness. Unexpectedly, after nectar removal bees visited a smaller proportion of flowers; this was a behavioural change not considered by the model, as in a previous experiment the number of visited flowers was independent of nectar volumes. Possibly, reduced flower visitation only occurs in completely rewardless flowers as described for deceptive species (Johnson et al., 2004), while small reward amounts as often found in *B. rapa* still stimulates complete flower visitation. Apart from this small inconsistency, our model covers the experimental results well and presents a mechanism that can explain honest floral signalling in *B. rapa*.

According to the model suggested here, honest signals must be associated with pollinator attraction and be under positive pollinator-mediated selection. Although bumble bees learning of honest signals emitted by *B. rapa* has been demonstrated in laboratory experiments (Knauer & Schiestl, 2015), it remained to be tested if pollinators are able to favour honest signals in nature where they are in contact with many different plant species and a large set of (potentially honest) floral signals. Here, we found that augmented phenylacetaldehyde emission increased bee attraction, but only when this compound was emitted as an honest signal in the plant population as a whole. This result demonstrates that a generalist pollinators' ability to identify and use a plant's honest floral signals extends to natural habitats. This is consistent with the findings from Kaesar *et al.* (2006), who showed that the manipulation of a floral signal affected bee attraction in a plant species where the signal was honest, but not in a species where the same signal was dishonest. Moreover, pollinator-mediated selection for larger honest signals appears to be a general phenomenon across various plant species (Armbruster et al., 2005; J. M. Gómez, 2003; Jose M. Gómez et al., 2008; Parachnowitsch et al., 2012).

In natural populations signal honesty might be lost as pollinators deplete rewards over time. This could undermine the maintenance of honesty as we found the pollinators' preference for the floral signal phenylacetaldehyde to depend on the signal being honest. High nectar replenishment rates and low pollinator abundance may contribute to the retention of honesty in plant populations. Further, various solitary and social bee species leave scent marks when visiting flowers, which can be recognized by con- and heterospecific species to avoid recently visited and likely

depleted flowers (Gawleta et al., 2005; Gilbert et al., 2001; Giurfa, 1993; Yokoi et al., 2007). Such scent marks could substantially contribute to the stability of honest signalling during proceeding reward depletion.

In line with the model, we also found pollinator-mediated selection for larger nectar volumes. Nectar volume was associated with visitation time, which affected the number of seeds that flowers developed. Similarly, in *Petunia axillaris* seed set was positively affected by hawkmoth visitation time which depended on the available nectar volume (A. Brandenburg et al., 2012; Anna Brandenburg et al., 2012). In the hummingbird pollinated *Ipomopsis aggregata*, corolla width honestly advertises nectar production and is under pollinator-mediated selection through an increased pollen export during single visits (Campbell et al., 1996). The relationship between nectar volume and an improved pollination during single visits may therefore apply to substantially different pollination systems.

In *B. rapa* the honest signals and seed set increased with soil fertilization. Incorporating these findings into our model showed that honesty is a stable strategy only if signals and seed development are resource limited (by the same resource). These results suggest that honest floral signalling requires high production costs (energetic/efficacy costs) to build the signal. It is known that petals can cause significant energetic costs (Schemske, 1978; Vasconcelos & Proença, 2015), and that petal size (or the size of bracts) is a frequently reported honest signal (Armbruster et al., 2005; Jose M. Gómez et al., 2008; M. Stanton & Young, 1994). Aromatic scent compounds like phenylacetaldehyde are metabolized from phenylalanine (Dudareva et al., 2013) and may therefore compete with other amino acid based metabolites which explains why the addition of nitrogen may increase the signal. In *Petunia hybrida*, both flower diameter and the phenylpropanoid eugenol increased with soil nitrogen concentration (Majetic et al., 2017) and seed development is nitrogen limited in many plant species (Łukowiak & Grzebisz, 2020; Sinclair & de Wit, 1975).

Our model, similar to the one by Cohen and Shmida (1993), assumes variable resource availability in flowers between plants of the same population. The variability of floral resources may be affected by species specific trade-offs as well as the resource availability in the environment. Low nutrient availability in the soil has been shown to reduce phenotypic variability in *Sinapis arvensis* (M. L. Stanton et al., 2000), possibly because individuals with the potential for a high nutrient assimilation

and allocation to flowers are not capable to express these properties. Although we did not explicitly model habitat-specific effects, the above suggests that honesty should evolve in habitats with intermediate to high nutrient availability, which is expected to modulate variation in nutrient limitation. Comparison of signal honesty in populations that vary in the availability of soil nutrients would allow to get insights into how these factors affect honest signalling.

Our study shows that resource availability impacts signal evolution in plants, an aspect that has received little attention so far. The mechanism we suggest to be responsible for floral honesty depends on the interaction between genetically controlled plant traits, pollinator behaviour and environmental factors. Honest floral signals should therefore only evolve in resource limited signals, plant species that rely on high visitation frequencies and long floral visitation times by pollinators and in environments with intermediate to high resource availability. Future investigations could usefully incorporate those aspects to deepen our understanding of signal evolution in plants.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTION

A.C. Knauer, F. P. Schiestl and H. Kokko conceived the ideas and designed methodology; A.C. Knauer collected and analysed the data; A.C. Knauer, F.P. Schiestl and H. Kokko wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available from the digital repository of the University of Zürich: <https://doi.org/10.5167/uzh-201676> (Knauer et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Appendix S1 [Additional verification of model]

Figure S1 [Model prediction on signal-seed correlation]

Table S1 [Model parameters]

FIGURES AND TABLES

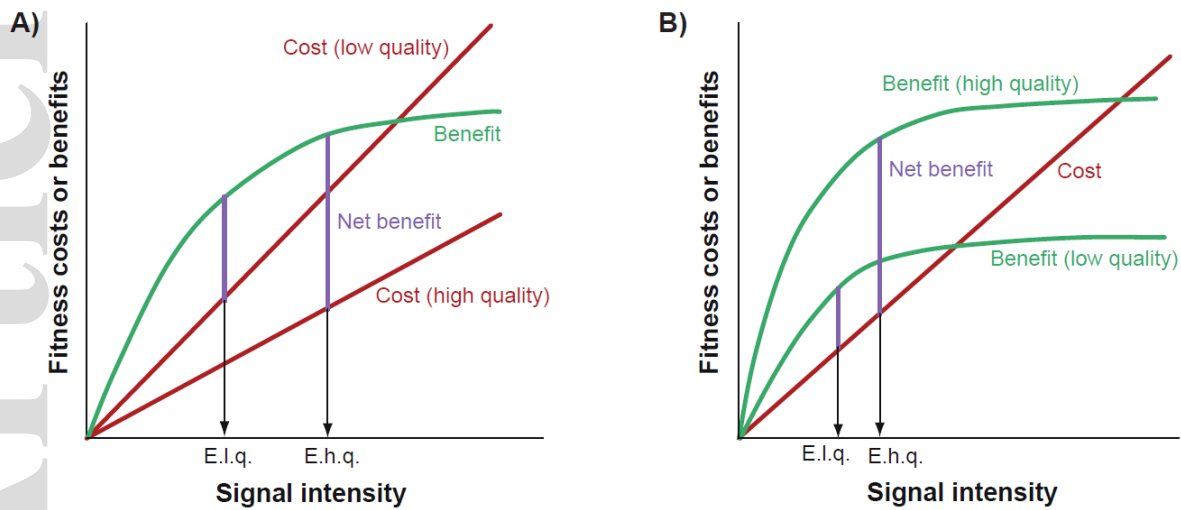


Figure 1 Honest signalling maintained by **A)** differential costs: low quality individuals have higher costs to produce large signals than do high quality individuals; **B)** differential benefits: low quality individuals have lower benefits from large signals than high quality individuals. The equilibrium signal intensities for low quality individuals (E.l.q) and high quality individuals (E.h.q.) are where the difference between benefit and cost is maximised. Adapted from Grose (2011).

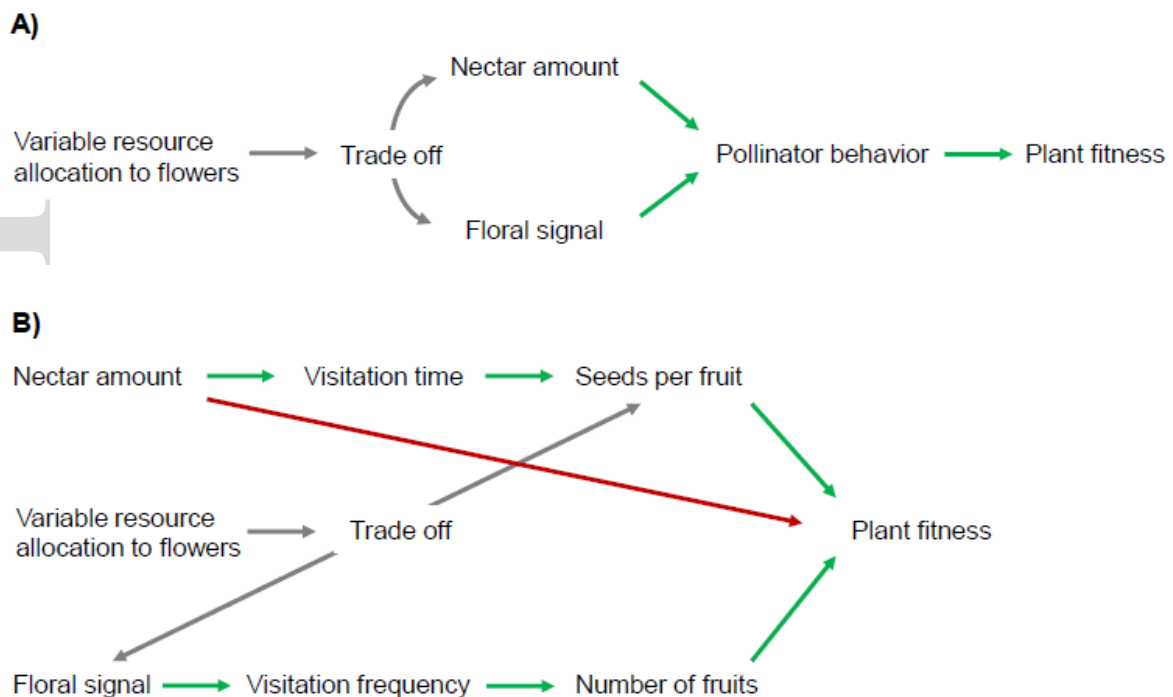


Figure 2 Mechanisms that can maintain the stability of honest floral signalling. Benefits are given as green lines, costs as red lines. **A)** Model by Cohen & Shmida (1993). The model assumes variable

resource availability in flowers, which explains how despite a trade-off between reward and floral signal, their across-individual relationship can be positively correlated. This arises when individuals optimize their fitness by allocating a specific proportion of the resource (p) to the signal and the rest ($1-p$) to the reward. **B)** Mechanisms that we consider to maintain honest floral signalling in *B. rapa*. The model includes associations that were found between floral traits, bumble bee behaviour and plant female fitness as well as the costs of nectar production and the resource limitation in honest signals and seed development. The model additionally assumes variable resource availability in flowers, which in combination with the trade-off between signals and seed development, causes a positive across-individual correlation between these two traits (similar to the correlation between nectar and signal in model A). Honest signalling arises as plants with low resource availability in flowers profit less from increased nectar volumes and the resulting longer pollinator flower visits because of their limited potential to develop large seed numbers (differential benefits, see Figure 1).

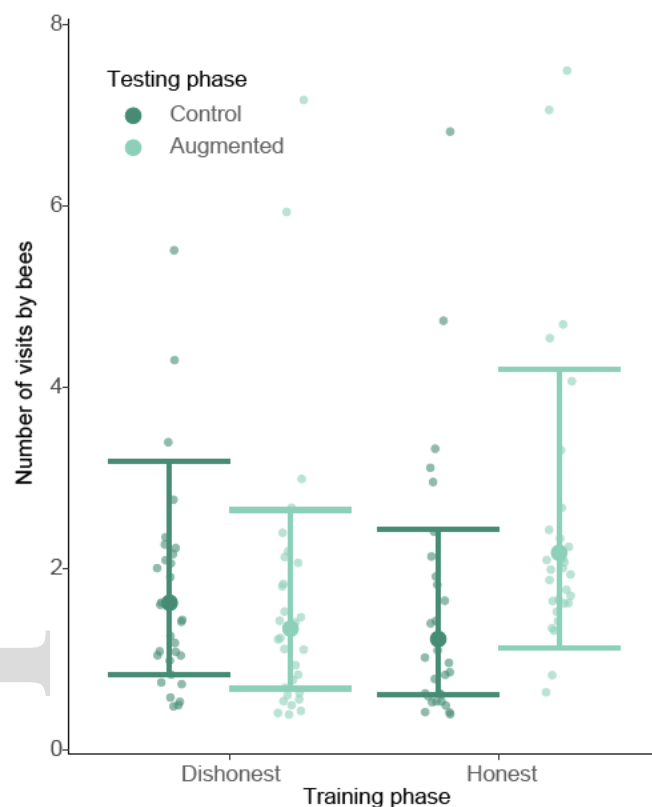


Figure 3 Predicted effect of augmented phenylacetaldehyde emission (\pm 95% CI) on pollinator attraction after bees had been foraging on plants emitting phenylacetaldehyde as an honest signal (training phase “honest”) or as a dishonest signal (training phase “dishonest”). Bees only preferred plants with augmented phenylacetaldehyde emission when they experienced this volatile as an honest signal previously.

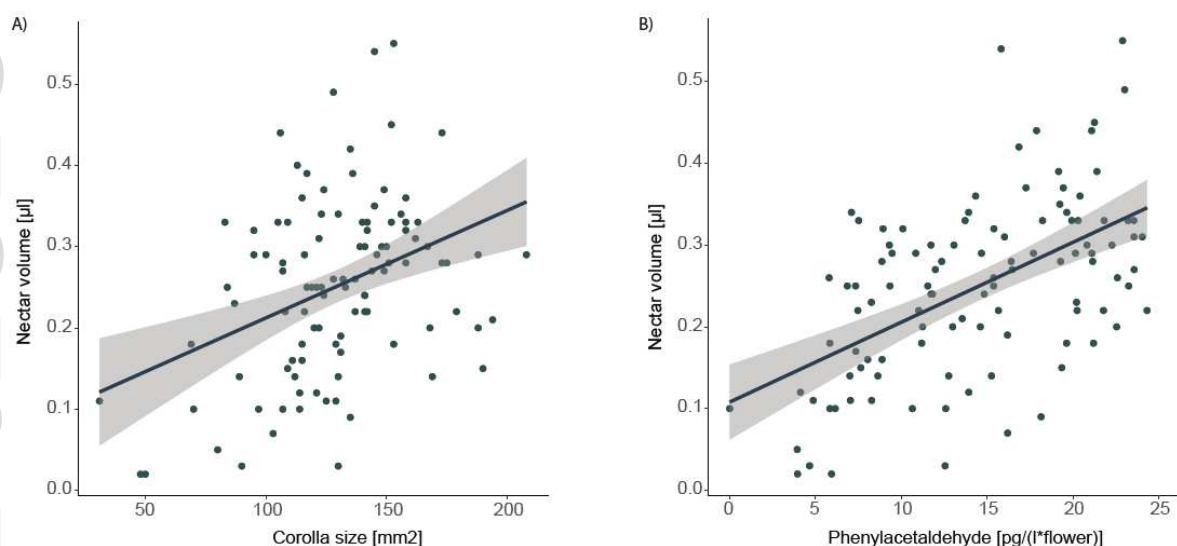


Figure 4 Honest signalling in *Brassica rapa*. Corolla size and the floral volatile phenylacetaldehyde are correlated with nectar volume.

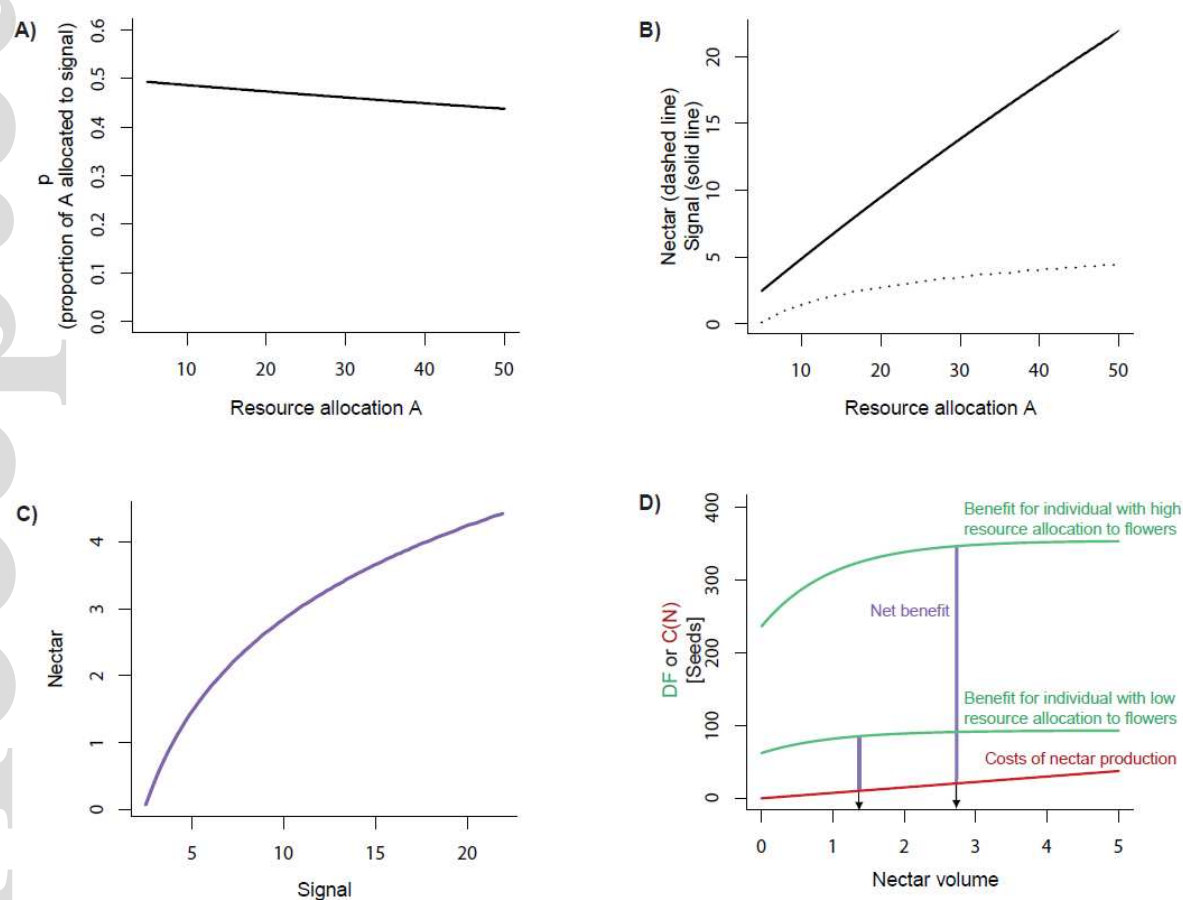


Figure 5 Results of the mathematical model. **A)** Proportion of resources p that plants optimally allocate to honest floral signals given a trade-off between signals and seeds. The optimal p decreases with increasing resource availability in flowers A . **B)** Optimal values of nectar N (dashed line) and signal S (solid line) for plant individuals with increasing resource availability in flowers A . **C)** The predicted signal-nectar association in a plant population with variable resource availability in flowers. **D)** The positive relationship between N and A , or S respectively, arises because of differential benefits of nectar production. The arrows show where the net benefit is maximal for individuals with low resource availability ($A = 10$) and individuals with high availability ($A = 20$) when both individuals signal at their optimal signal level (4.86 and 9.48 for $A = 10$ and 20, respectively).

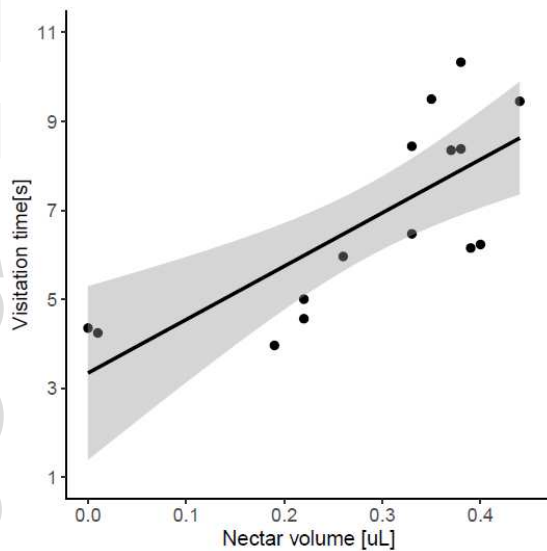


Figure 6 Association between flower visitation time and nectar volume (± 95 CI) showing that bees spend more time on flowers with large nectar volume.

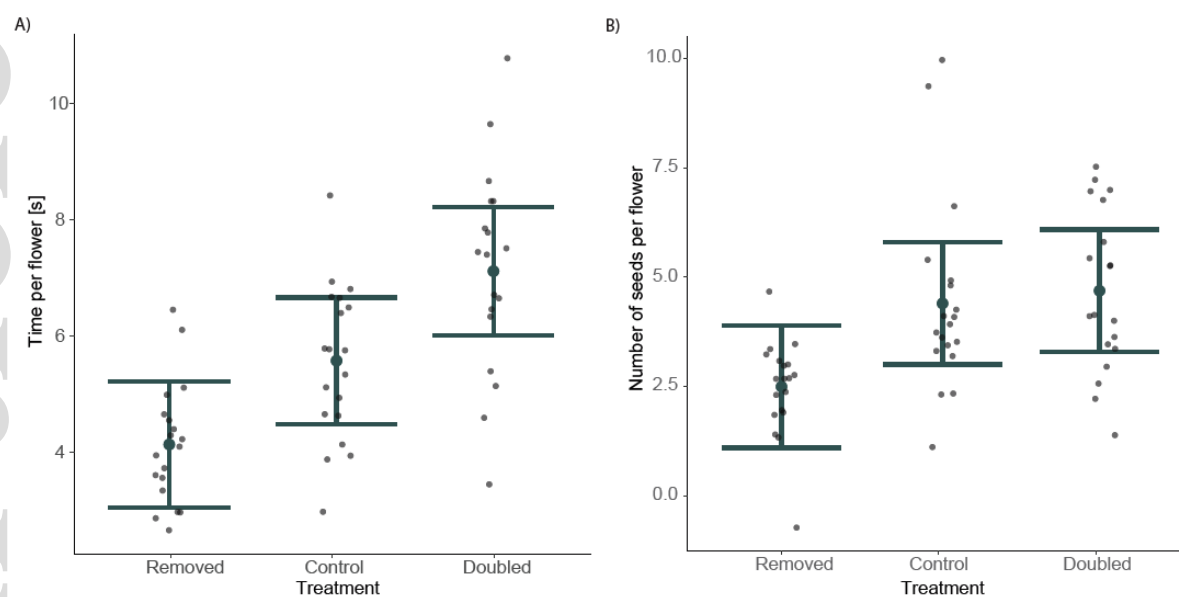


Figure 7 Predicted effect of nectar manipulation (\pm 95% CI) on **A)** visitation time by bumble bees; **B)** female plant fitness. Nectar removal reduced the flower visitation time by bumble bees while nectar addition increased it. Plant fitness was reduced after nectar removal but not affected by nectar addition.

Table 1 Directional selection on floral traits.

Floral trait	β	t	P
Nectar volume	0.16 ± 0.10	2.315	0.023
Corolla size	0.17 ± 0.09	4.804	< 0.001
Phenylacetaldehyde	0.19 ± 0.10	2.507	0.014
Flower number	0.41 ± 0.09	3.568	< 0.001

Significant results are given in bold